



Protective role of resveratrol on testicular germ cells in mice with testicular toxicity

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Cite this article as: Özatik FY, Özatik O, Yiğitaslan S, Çengelli Ünel Ç, Erol K. Protective role of resveratrol on testicular germ cells in mice with testicular toxicity. Turk J Urol 2017; 43(4): 444-50.

ABSTRACT

Objective: The aim of the present study was to investigate the possible beneficial effects of resveratrol in mice subjected to vinyl cyclohexene dieposide (VCD) -induced testicular toxicity.

Material and methods: A total of thirty- six Swiss albino male mice aged 28-days were used in the present study. The study was composed of two stages where mice which received or did not receive VCD (320 mg/kg/day) were administered resveratrol. The animals were assigned into control and resveratrol-treated groups in the first stage and into groups of VCD- and VCD+resveratrol-treated groups in the second stage. At the end of the experiments, relative testicular weight (TW/BW) and dry/wet weight of testis (TDW/TWW) were calculated. Histological analysis by hematoxylin and eosin (H&E) staining and immunohistochemical staining by BAX and Bcl-2 were performed. Serum testosterone, LH and FSH levels were measured by a commercially available ELISA kit.

Results: Resveratrol caused a dose-dependent increase in TW/BW and decrease in TDW/TWW ($p<0.05$). Resveratrol at a dose of 20 mg/kg resulted in an improvement in testosterone, LH and FSH levels in mice with VCD-induced testicular toxicity ($p<0.001$). Resveratrol also improved apoptotic index and epithelial cell height of testicular seminiferous tubuli significantly after VCD exposure ($p<0.001$).

Conclusion: Results of the present study suggest that resveratrol can be used as a protective and/or therapeutic agent particularly for cases with male infertility caused by testicular toxicity.

Keywords: Mice; resveratrol; testis; toxicity.

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Submitted:
10.12.2016

Accepted:
18.04.2017

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Introduction

Resveratrol (RES) (3,5,4'-trihydroxystilbene) is a natural phytoalexin that is found particularly in red wine and peanuts.^[1] There are two RES forms ie. -cis and -trans with all forms being present in a greater amount in red grapes compared to the green grapes.^[2] The -trans form of the RES has been reported to affect estrogen receptors which are also important in male reproductive system. Furthermore, chemical structure of RES is similar to that of diethylstilbestrol.^[1,3] In recent years, several *in vivo* and *in vitro* studies have suggested that RES protects the spermatocytes against lipid peroxidation as

well as increases testicular sperm count and sperm motility.^[4] RES has also been reported to increase the sperm production and to decrease germ cell apoptosis.^[5] Furthermore, it is well known that RES is protective against environmental toxins.^[6] It is also a remarkable antioxidant agent with some previous reports suggesting a more potent antioxidant effect than well-known antioxidants Vitamin C and E.^[7] Another study found that the grape seed extract containing RES has protected the glial cells from oxidative stress.^[8] The antioxidative effects of RES have been attributed to its inhibition of cyclooxygenase transcription in DNA polymerase activity and ribonucleotide reductase.^[9]

Vinylcyclohexene diepoxide (VCD) is a reactive compound that is commonly used in paints and adhesives. Thus, many individuals working in this branch of industry are exposed to high amounts of this compound.^[10] VCD is known as an oncogenic environmental pollutant.^[11] It is the only chemical causing damage in the primordial and primary follicles of rat and mice oocytes.^[12] However, to our knowledge, there is only one study reporting that VCD causes a dose- and time-dependent testicular toxicity in male mice, which may be attributed to the apoptotic effect of VCD.^[10,13]

In the light of all these data, present study was designed to investigate the testicular effects of RES in mice with and without VCD-induced testicular toxicity.

Material and methods

A total of 36 Swiss albino male mice aged 28-days were used in the present study. All animals were kept in cages. The animals were maintained under standard conditions of temperature ($25\pm 2^\circ\text{C}$), humidity ($65\pm 5\%$) and light (12/12 h light/dark) and were fed with standard food pellets and had free access to water. All animal experiments were conducted according to the guidelines for the care and use of laboratory animals and the approval was obtained from the local Ethics Committee for the Care and Use of Experimental Animals of Eskisehir Osmangazi University (date: 03/03/2011 and number: 189).

The study was composed of two stages using RES with and without exposure to VCD, respectively. The animals were assigned into the control and RES-treated groups in the first stage and VCD- and VCD+RES-treated groups in the second stage.

Animal experiments

In the first stage of the study, mice were only treated with RES (Sigma- Aldrich, St. Louis, MO, USA) without exposure to VCD. The groups of Control, RES2, RES10 and RES20 included 6 mice in each ($n=6$). After 1 week of acclimation period, RES2, RES10 and RES20 groups were treated with 2, 10 and 20 mg/kg of RES by oral gavage, while control group received equal volume of dimethyl sulfoxide (DMSO) (Sigma- Aldrich, St. Louis, MO, USA). In previous studies, it was observed that RES at a dose of 20 mg/kg did not produce systematic toxicity.^[7] RES treatment was continued for 3 days with 24- hour intervals. Twenty-four hours after the last dose of RES, body weights (BW) of the animals were determined and recorded, blood samples were withdrawn from the heart under general anesthesia and animals were killed by overdose of anesthesia. Serum samples obtained by centrifugation of blood samples at 3000 rpm for 3 min were stored at -20°C until analysis. Both testes of mice were removed and weighed to determine the wet

weight of testis (TWW). One testis was used for the histological analysis and the other was stored at -80°C until dry weights of testes (TDW) were estimated. For the determination of TDW, testicular tissues were dried at 60°C for 12 hours in an oven.

In the second stage of the study, 320 mg/kg of VCD (Fluka Chemical Co. Ltd, Switzerland) was injected intraperitoneally for 10 days.^[10] After 10 days of VCD treatment, all animals except from those in the VCD group were treated with 20 mg/kg RES by oral gavage at a dose that was found to be effective in the first stage. Twenty-four hours after the third dose of the treatment, BWs of the animals were determined and recorded, blood samples were withdrawn from the heart under general anesthesia and animals were killed by overdose of anesthesia. Serum samples obtained by centrifugation of blood samples at 3000 rpm for 3 min were stored at -20°C until analysis. Both testes of mice were removed and weighed to determine the TWWs. One testis was used for the histological analysis and the other was stored at -80°C until determination of TDW. For the determination of TDW, testis tissues were dried at 60°C for 12 hours in an oven.

The ratio of testis weight to body weight (TW/BW) was calculated by dividing the testis weight in milligrams to the body weight of animals in grams. The ratio of dry to wet weight of testis (TDW/TWW) was calculated by dividing the wet weight of testis in grams by the dry weight of the testis in grams.

Histological evaluation

Testicular tissues were fixed in 4% paraformaldehyde and then embedded in paraffin blocks. Sections cut at $5\ \mu\text{m}$ thickness were stained with hematoxylin and eosin (H&E), BAX and Bcl-2. The sections were then viewed under light microscope to determine the histological changes.

In H&E stained sections, epithelial cell height of testicular seminiferous tubuli (ECHST) was measured and recorded. Johnsen scoring system was used for the grading of testicular toxicity.^[14] In BAX and Bcl-2 stained sections, mean apoptotic germ cell count was determined for each tubule by evaluating 25 tubules per testis.

Biochemical evaluation

Serum levels of testosterone, LH and FSH were measured by using commercially available rat ELISA kits (Cayman, USA) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed by using IBM Statistical Package for the Social Sciences (IBM SPSS Statistics; Armonk, NY, USA) 20.0 package program. The distribution pattern of data was evaluated by Shapiro Wilk's test. The intergroup

comparisons were performed by using One Way ANOVA test. Exact digit post hoc test was used to identify the groups with difference. Data were expressed as mean±SEM. P<0.05 was considered as statistically significant.

Results

In mice treated only with RES (RES2, RES10 and RES20), TW/BW ratio was significantly higher compared to the control group (p<0.05) (Table 1). However, any significant difference was not observed for pre-treated group with VCD.

In 3 groups treated with only RES, TDW/TWW was found to be significantly decreased in RES2 and RES20 groups compared to the control group (p<0.05) (Table 1). However, any significant difference was also not observed for pre-treated group with VCD.

Serum testosterone level was significantly decreased in RES2 and RES10 groups compared to the control group (p<0.001) (Figure 1). Although not being significant, testosterone level was slightly decreased in the RES20 group compared to the control group. On the other hand, serum testosterone was significantly lower in VCD-treated animals compared to the controls (p<0.001) but significantly higher in VCD+RES20 group compared to VCD-treated animals (p<0.001), suggesting that 20 mg/kg RES treatment relieved testicular toxicity and increased testosterone secretion.

Serum LH level was significantly increased in RES20 and VCD + RES20 group compared to the control group (p<0.001) (Figure 2). In addition, serum LH level was significantly higher in VCD+RES20 group compared to VCD group (p<0.05) (Figure 2).

Serum FSH level was found to be significantly decreased in VCD-treated animals compared to the control animals (p<0.05) with a significant increase in VCD+RES20 group compared to the VCD group (p<0.05) (Figure 3).

Table 1. Testicular weights

	Relative testicular weight, mg/g	Dry/wet testicular weight, g/g
Control	5.35±0.226	0.186±0.006
RES2	6.6±0.7*	0.131±0.017*
RES10	7.395±0.49*	0.218±0.038
RES20	6.818±0.45*	0.0173±0.007*
VCD	5.73±0.28	0.156±0.011
VCD+RES20	5.44±0.4	0.158±0.0058

Values are expressed as mean±SEM. Differences from the control group (*). *p<0.05. VCD: vinylcyclohexene diepoxide; RES: resveratrol

Histological results

Johnsen scores

Johnsen score was significantly lower in VCD and VCD+RES20 groups compared to the control group (p<0.05) (Table 2). Although not being statistically significant, the scores were slightly higher in VCD+RES20 group compared to VCD group.

Epithelial cell height of testicular seminiferous tubuli (ECHST)

Epithelial cell height of testicular seminiferous tubuli was significantly higher in RES10 and RES20 groups compared to the control group (p<0.001) (Table 2). However, in VCD group, ECHST was significantly lower compared to the control group (p<0.001). In animals treated with VCD+RES20, ECHST was found to be significantly lower compared to the control group but significantly higher relative to the VCD group (p<0.001 for each).

Apoptotic cell count in testicular tubules

Apoptotic cells were counted in testicular tubules and found to be significantly higher in the VCD group compared to the control group (p<0.001) with a significant decrease with VCD+RES20 treatment (p<0.001) (Table 2, Figure 4 and 5).

Discussion

Vinylcyclohexene diepoxide is a reactive compound and many people working in an industrial area are exposed to high amounts of this compound.^[10] It is commonly used in chemical industry as well as in synthetic yarn and carpet manufacturing. VCD is a mutagen for Salmonella bacteria system and leads to germ cell destruction by attaching DNA and preventing DNA synthesis.^[10] In a study by Hooser et al.^[5] VCD has been

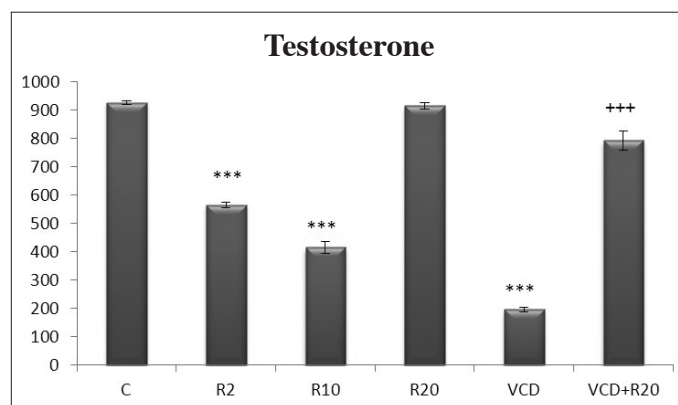


Figure 1. Serum testosterone

(*): different from the control (+): different from the VCD group (**): p<0.001 (+++): p<0.001. C: control; VCD: vinylcyclohexene diepoxide

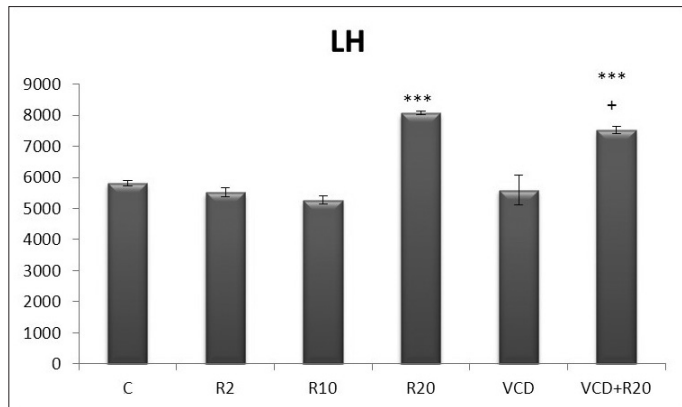


Figure 2. Serum LH levels

(*): different from the control (+): different from the VCD group (**): $p < 0.05$ (***): $p < 0.001$ (++++): $p < 0.001$. C: control; LH: luteinizing hormone; VCD: vinylcyclohexene diepoxide

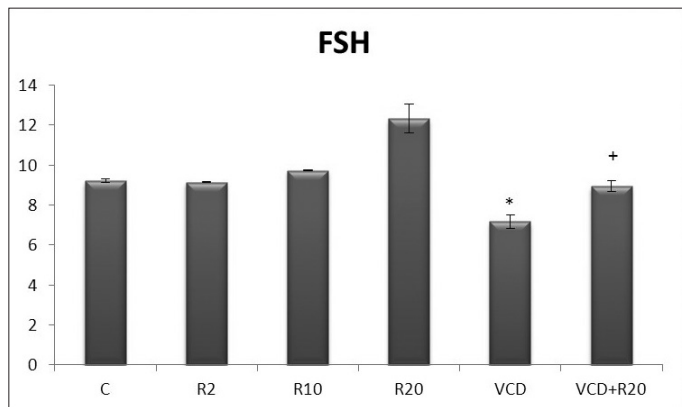


Figure 3. Serum FSH level

(*): different from the control (+): different from the VCD group (**): $p < 0.05$ (++++): $p < 0.05$. C: control; LH: luteinizing hormone; FSH: follicle stimulating hormone; VCD: vinylcyclohexene diepoxide

suggested to result in testicular toxicity in male mice through impairing DNA synthesis and cell replication (differentiated spermatogonia and pre-leptotene spermatocytes).

Resveratrol is a natural antioxidant that is commonly found in peanuts, grapes and beverage made from grapes. In addition to its well-known antioxidant capacity, RES has also been suggested to have vasorelaxing effects, to regulate lipoprotein metabolism, to inhibit thrombocyte aggregation and to have protective and therapeutic effects against cancer.^[15,16] All these data suggest that RES may be a molecular and/or cellular trigger which may also be true for estrogen system.^[15,17] The structural similarity found between trans-resveratrol, diethylstilbestrol and estradiol has led researchers to investigate the possible estrogen modulating effects of RES.^[15] In the present study, the protective effect of RES on testicular germ cells was investigated on

Table 2. Effects of resveratrol treatment on VCD-induced testicular toxicity

	Johnsen Scores	Apoptotic index	Tubuloseminiferous epithelial height
Control	9.93±0.033	0.683±0.0606	56.417±0.232
RES2	9.76±0.021	0.683±0.0606	56.433±0.192
RES10	9.78±0.030	0.567±0.0645	57.8±0.157 [#]
RES20	9.8±0.036	0.483±0.0651	59.133±0.133 [#]
VCD	9.3±0.13*	6.667±0.118 [#]	44.783±0.167 [#]
VCD+RES20	9.48±0.07*	4.567±0.107 ^{#,&}	50.667±0.138 ^{#,&}

Values are given as mean ±SEM. Differences from the Control (*) or VCD group (+). * $p < 0.05$; [#] $p < 0.001$; [&] $p < 0.001$. VCD: vinylcyclohexene diepoxide; RES: resveratrol

animals with and without testicular toxicity. For this purpose, TW/BW, TDW/TWW, histological and immunohistochemical analysis, and serum testosterone, LH and FSH levels were evaluated. Although all three doses of RES significantly increased TW/BW in animals without testicular toxicity, this was not true for animals with testicular toxicity. Similar findings were also found for ECHST which was found to be significantly increased in RES10 and RES20 groups compared to the control animals but was not significantly changed in VCD+RES20 group compared to the VCD group. Testicular weight, which was increased in animals without testicular toxicity but unchanged in animals with testicular toxicity, may indicate the variability in ECHST. Sharma et al.^[18] have investigated the role of RES in the treatment of testicular toxicity in Wistar rats induced by cypermethrin that is an insecticide from the group of synthetic pyrethroid. In parallel with our findings, the authors have reported significantly decreased TW by cypermethrin which returned to normal control values with RES treatment.

The ratio of dry to wet weight of testis was found to be significantly decreased in RES2 and RES20 groups. However, it was not true for VCD-treated animals, suggesting that only fluid retention in testis was antagonized by RES through a process called rehydration. In a previous study, CP336,156, a nonsteroidal estrogen agonist/antagonist agent has been reported to have rehydrating effect on uterine tissue.^[19] RES has been also suggested to have estrogen modulatory effects.^[20] Thus, RES may also have a similar effect on testicular tissue.

Testosterone is essential for structural morphology and physiology in seminiferous tubules for spermatogenesis.^[21] Juan et al.^[22] have found that treatment with 20 mg/kg RES for 90 days increased serum testosterone and gonadotropin levels and decreased tubular density and sperm counts in rats. Accordingly, in the present study evaluating the effects of 3 days of 20 mg/kg RES on mice exposed to VCD-induced testicular toxicity, significantly increased testosterone, FSH

and LH levels suggesting that RES might improve the VCD-induced gonadal dysfunction in mice. Indeed, in two studies performed in rats evaluating the therapeutic potential of RES in testicular toxicity models induced by doxorubicin and cypermetrin, testosterone, LH and FSH levels have been found to increase significantly.^[18,20] Juan et al.^[22] have reported that RES stimulated the gonadotropin secretion which is a major endocrine regulator in spermatogenesis. In that study, serum FSH and LH levels -which stimulates spermatogenesis in tubules and testosterone production in Leydig cells, respectively- were also found to increase significantly in RES-treated animals compared to the control group.^[22] The authors concluded that RES may induce pituitary stimulation and -improve testicular function. Accordingly, the results of the present study also support this conclusion. Since hypothalamic- pituitary- gonadal endocrine regulation in men is complex and related to both estradiol and testosterone.^[23,24] These results may suggest that RES may improve gonadal function after exposure to an agent causing testicular toxicity.

In the present study, VCD induced several histological changes including irregularity in basal membrane and germinal epithelia of seminiferous tubules, degeneration, vacuolization and germ cell loss. Johnsen scores also significantly decreased in VCD-treated animals, representing the histological changes induced by VCD in mice. Although not being significant, RES treatment after VCD injection resulted in a slight decrease in Johnsen scores. It can be suggested that RES at daily doses of 20 mg/kg improves testicular damage induced by VCD. Results of immunohistochemical studies were also in parallel to these findings. Similarly, in a study by Soylemez et al.^[25] it was determined that RES at a dose of 10 mg/kg increased the Johnsen scores to the control levels. The results in this study support our results.

In the histological analysis, VCD has been found to cause a decrease in ECHST and an increase in apoptotic cell count which are possibly resulted from the impairment of spermatogenesis.^[10] This kind of effect induced by VCD is also seen with the use of antineoplastic agents including adriamycin, bleomycin, cyclophosphamide and doxorubicin.^[10,21] In the study by Hooser et al.^[5], VCD has been reported to cause testicular germ cell necrosis in mice. The authors have suggested that VCD caused testicular toxicity by impairment of DNA synthesis and replication (differentiated spermatogonia and preleptone spermatocyte).^[5] A great proportion of spermatozoa membrane is composed of saturated fatty acid with a low level of antioxidant enzymes in cytoplasm. Oxidative stress that is associated with denaturation and fragmentation has a great role in abnormal sperm production.^[21,26] Some previous studies have suggested that doxorubicin also induces DNA fragmentation and chromosomal damage^[21,27,28], leading to increased

oxidative stress and increased number of mobile sperm count and abnormal sperm percentage.^[29] In a model of *Drosophila melanogaster*, vinyl cyclohexene (VCH) exposure for 5 days had increased reactive oxygen and nitrogen species (ROS and RNS), mRNA gene expression of superoxide dismutase, Nrf-2 and MAPK-2, changed antioxidant enzyme levels, and inhibited of δ -ALA -D and AChE activities.^[13] Conduction of a recent study is important to support the toxic effects of both VCH and VCD.

Apoptosis plays a great role in toxicity. In our study, immunohistochemical staining by Bax and Bcl2 was used to demonstrate the apoptosis. However, RES treatment at daily doses of 20 mg/kg decreased the apoptotic cell count induced by VCD. RES is known to prevent the lipid peroxidation and DNA damage induced by oxidative stress.^[30] Accordingly, RES has also been suggested to decrease the oxidative stress in seminiferous tubules and to increase sperm maturation.^[31]

Results of the present study suggest that RES can be used as a protective and/or therapeutic agent particularly for cases of male infertility caused by testicular toxicity. Infertility is a great health problem and has several different causes. In the present study, daily treatment with RES in mice stimulated hypothalamo-pituitary-gonadal axis without any adverse effect, and improved the VCD induced testicular damage by its antioxidant effect.

Study limitations

There are some limitations of this study. Western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) must have been done in order to investigate the protein and gene expression of spermatogenic cell membrane. However these analyses could not be performed with the current laboratory conditions. Thus these analyses were thought to be added up in our further studies.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of The Care and Use of Experimental Animals of Eskisehir Osmangazi University (Date: 03/03/2011 and number: 189).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – F.Y.Ö.; Design – F.Y.Ö., K.E.; Supervision – F.Y.Ö., K.E., S.Y., O.Ö.; Resources – F.Y.Ö., Ç.Ç.Ü., O.Ö.; Materials – F.Y.Ö., O.Ö.; Data Collection and/or Processing – F.Y.Ö.; Analysis and/or Interpretation – F.Y.Ö., O.Ö., S.Y., Ç.Ç.Ü., K.E.; Literature Search – F.Y.Ö., O.Ö., S.Y.; Writing Manuscript – F.Y.Ö., O.Ö.; Critical Review – F.Y.Ö., O.Ö., S.Y., Ç.Ç.Ü., K.E.; Other – F.Y.Ö., O.Ö., K.E.

Conflict of Interest: No conflict of interest was declared by the authors.

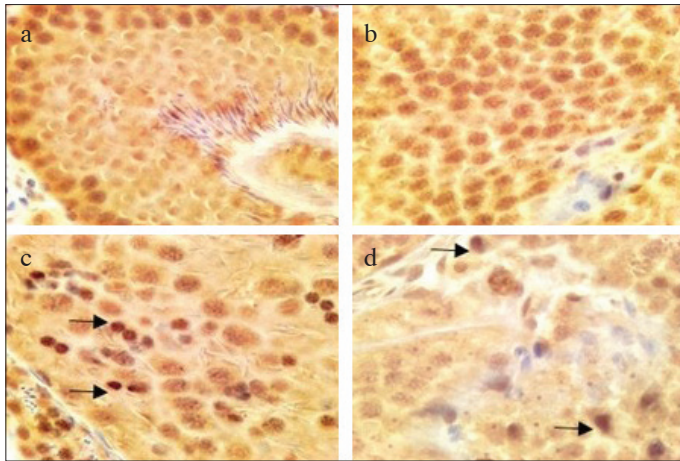


Figure 4. a-d. x 40, Bcl2. (a) control, (b) RES 20, (c) VCD indicates the increased number of apoptotic germ cell (→), (d) VCD+RES 20 shows less number of apoptotic germ cells (→) when compared to VCD group
Bcl2: anti-apoptotic gene Bcl2; RES: resveratrol; VCD: vinylcyclohexene diepoxide

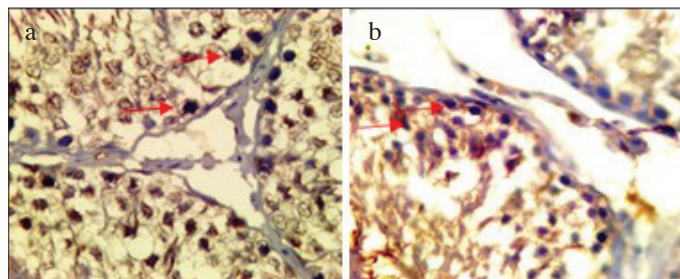


Figure 5. a, b. x40, Bax, (a) VCD showing higher numbers of apoptotic germ cells (→), (b) VCD+RES 20 indicating lesser number of apoptotic germ cells compared to VCD group (→)
Bax: Bcl2- associated; RES: resveratrol; VCD: vinylcyclohexene diepoxide

Financial Disclosure: The authors declared that this study has received no financial support.

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